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# Effect of short-term versus prolonged freezing on freeze-thaw injury and post-thaw recovery in spinach: Importance in laboratory freeze-thaw protocols

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**Effect of short-term versus prolonged freezing on freeze-thaw injury and  
post-thaw recovery in spinach: Importance in laboratory freeze-thaw  
protocols**

by

**Kyungwon Min**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirement for the degree of  
**MASTER OF SCIENCE**

Major: Horticulture

Program of Study Committee:  
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Ames, Iowa

2014

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## ABSTRACT

Spinach (*Spinacia oleracea* L.) is a cool-season vegetable crop which can be damaged by intermittent spring frost that is needed to better understand freezing tolerance of spinach. This thesis focused on the physiology of spinach's freezing tolerance through duration of freezing, including an ability of recovery, using freeze-thaw injured spinach tissues exposed to various durations of freezing at two sub-lethal temperatures. Various physiological parameters [electrolyte leakage, Fv/Fm ratio (efficiency of PSII), MDA test (an indicator of lipid peroxidation), and histochemical detection of ROS (i.e. superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ))] were quantified. Based on the  $LT_{50}$  of spinach (approximately  $-5.5\text{ }^{\circ}\text{C}$ ),  $-4$  and  $-4.5\text{ }^{\circ}\text{C}$  were selected as two sub-lethal temperatures for prolonged freezing test, but only  $-4.5\text{ }^{\circ}\text{C}$  for post-thaw recovery test. Enhanced ion-leakage, increased MDA content, accumulated ROS and decreased Fv/Fm ratio were observed in freeze-thaw injured spinach tissues subjected to four duration (0.5, 3.0, 5.5 and 10.5 h) of freezing at both  $-4$  and  $-4.5\text{ }^{\circ}\text{C}$ ; however, all measurements were shown to be worse at  $-4.5\text{ }^{\circ}\text{C}$  than  $-4.0\text{ }^{\circ}\text{C}$  during four durations of freezing. For the post-thaw recovery test, spinach tissues frozen for relatively short-term duration of freezing at  $-4.5\text{ }^{\circ}\text{C}$  was recoverable during post-thaw periods, but was irrecoverable when stressed by a longer duration of freezing at the same temperature. In summary, depending upon duration of freezing, temperatures interpreted as sub-lethal based on  $LT_{50}$  (short-term freezing assays) could be actual lethal. Therefore, "duration of freezing" should be considered as one of critical factors influencing on plant freeze-thaw tolerance when using artificial freeze-thaw protocols.

## CHAPTER 1. GENERAL INTRODUCTION

### INTRODUCTION

#### *Cold stress and freeze-thaw process in plant tissues*

Cold stress can cause substantial economic losses to farming industry, which gives rise to many studies towards understanding and improving of the biology of plant cold hardiness. Cold stress can be classified in two types: 1) a chilling stress which occurs below a window temperature, usually between 10~15 °C and 2) a freezing stress, which happens below 0 °C (Levitt, 1980). Due to innate characteristics such as poikilotherms and sessile, plants are unavoidable to be exposed to low temperatures and therefore, plants have two ways to resist low temperature stress: 1) avoidance (preventing any ice formation within plant tissues) and 2) tolerance (minimizing the injurious effects as ice crystals formed around extracellular space) (Levitt, 1980); tolerance to low temperature stress is the main mechanism since they are essentially unable to avoid an environmental freezing stress.

During a natural frost episode, plant tissues are slowly cooled down in sub-freezing ranges at the rate of  $1 \sim 2 \text{ }^{\circ}\text{C h}^{-1}$  (Levitt, 1980; Steffen et al., 1989), leading to ice crystals formed around extracellular (apoplast) space within plant cells since the extracellular fluid generally has a higher freezing point, a lower concentration than the intracellular fluid. Consequently, a water potential gradient is established to drive water efflux from intracellular space to apoplast, thus resulting in enlargement of extracellular ice crystals until the water remaining inside the cell reaches a thermodynamic equilibrium with the extracellular ice, and hence, cell desiccation occurred (Levitt, 1980; Guy 1990; Arora and Palta, 1991; Ashworth, 1992; Thomashow, 1999; Xin and Browse, 2000). After thawing with temperatures going up,

plant cells are expanded to return to an original shape, in which various physiological dysfunctions are observed.

### ***Various symptoms of freeze-thaw injury in plant tissues***

After freeze-thaw cycles, plant tissues show diverse injuries at the cellular level. The plasma membrane has generally been regarded as the primary site of freezing injury within plant cells (Levitt, 1980; Palta and Li, 1980; Steponkus, 1984; Yoshida, 1984; Uemura et al., 2006); such as lamellar-to-hexagonal-II, fracture jump lesions, and expansion-induced-lysis are included in freeze-induced membrane damages following the critical cellular desiccation (Steponkus, 1984; Steponkus et al., 1993; Uemura et al., 1995; Mahajan and Tuteja, 2005). Freeze-thaw stress to plant tissues is also believed to induce the decline in the rate of photosynthesis due to the decreased enzymatic reaction of Calvin cycle that can ultimately lead to photon flux in excess of that is required in the PSII reaction center or for assimilation of CO<sub>2</sub> (Rizza et al., 2001; Hopkins and Huner, 2003). If this excess light energy is not dissipated as heat or fluorescence, the PSII reaction center could be inhibited that can result in decreased rate of photosynthesis and accumulation of reactive oxygen species (ROS) (Öquist and Huner, 2003; Adams et al., 2004).

Kendall and Mckersie (1989) reported that accumulated ROS is observed in freeze-thaw injured plant tissues. ROS are partially reduced forms of atmospheric oxygen; they are generally generated from the excitation of O<sub>2</sub> to produce singlet oxygen (O<sub>2</sub><sup>•</sup>) or from move of one, two or three electron to O<sub>2</sub> to generate, respectively, a superoxide radical (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or a hydroxyl radical (HO<sup>•</sup>). ROS, if not scavenged by antioxidant system as either enzymatic or non-enzymatic, are substantially toxic to various organelles within plant cells and hence, oxidative stress to protein, DNA, and lipid (Foyer et al., 1994; Rossini et al., 2006). Sharma et al., (2012), for example, noted that if ROS level reached to

above threshold at which plant do not handle with, enhanced lipid peroxidation occurs in both cellular and various organelles membrane that ultimately aggravate the oxidative stress by production of lipid-derived radicals which can react with and damage proteins and DNA. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acid in phospholipid, thus indicating that increase in lipid peroxidation under cold stress condition may be potentially linked to accumulation of ROS and enhanced ion-leakage (Yong et al., 2008; Liu et al., 2013)

### ***Various methods to measure plant freeze-thaw injury***

#### ***(1) Plasma membrane injury measured by electrolyte ion-leakage***

Under a natural condition, freeze-thaw stress to plasma membrane could be studied in two ways: 1) the structural perturbations through disruption of lipoprotein structure, and 2) functional damage to, for example, membrane transport functions reflected in altered membrane permeability, thus resulting in enhanced ion-leakage (Palta et al., 1977); it has been believed that the maintenance of their integrity and stability is the main component of freeze-thaw stress (Whitlow et al., 1992). Therefore, measuring the membrane permeability via the electrolyte ion-leakage, first demonstrated by Dexter et al. (1932), is widely and routinely employed to evaluate the extent of freeze-thaw injury in excised plant tissues. Most generally, percent injury and percent adjusted injury from raw ion-leakage data are used to determine the  $LT_{50}$  as plotting a sigmoidal curve, which is fitted as Gompertz function (Lim et al., 1998); also percent adjunct injury is significantly correlated to visual estimates (e.g. the extent of water soaking).

#### ***(2) Efficiency of photosystem II (photosynthesis) measured by a chlorophyll fluorometer***

Decrease in photosynthesis rate is also one of the common symptoms following freeze-thaw stress within plant tissues (e.g. injury in chloroplast membrane) (Hincha and



Schmitt, 1992). Photosynthetic CO<sub>2</sub> fixation of protoplasts is the most freezing sensitive reaction in which photosynthetic electron transport and photo-phosphorylation could be inhibited (Rumich-Bayer and Krause, 1989); additionally, accumulated ROS would disrupt D1 protein, a component of PSII (Krieger-Liszkay, 2005).

Light energy absorbed by green tissues, most typically, experiences three fates: 1) photochemistry energy which is actually used for photosynthesis, 2) dissipated as heat, and 3) re-emitted as chlorophyll fluorescence; these three processes occurred in competition in which increase in one of three energy results in decrease in the yield of the other two (Maxwell and Johnson, 2000). In recent years, chlorophyll fluorescence analysis, using a chlorophyll fluorometer, has been employed to evaluate the efficiency of photosystem II in response to various environmental stresses, including freeze-thaw stress (Maxwell and Johnson, 2000; Baker and Rosenqvist, 2004). Chlorophyll fluorescence estimates the fate of excitation energy as nondestructively and this technique could provide a reliable, sensitive, and quick assessment of plant freeze-thaw tolerance in various plant species (Binder and Fielder, 1996; Rizza et al., 2001; Percival and Henderson, 2003; Equiza and Francko, 2010).

The chlorophyll fluorometer measures various parameters such as  $F_o$  (minimal fluorescence) and  $F_m$  (maximal fluorescence), thereby calculating  $F_v$  (variable fluorescence); thus, the efficiency of PSII is able to be calculated with the following formula:  $F_m - F_o / F_m$ , generating  $F_v/F_m$  ratios; most generally, under optimal conditions various plant species present around 0.83 values ( $F_v/F_m$  ratios) (Demmig and Björkman, 1987; Maxwell and Johnson, 2000).

### ***(3) Detection of reactive oxygen species (ROS) measured by histochemical staining***

Freeze-thaw stress accelerates generation of reactive oxygen species (ROS) such as superoxide ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $OH^{\bullet}$ ), thus inducing

oxidative stress within plant cells (Kendall and McKersie, 1989). ROS are constantly generated through aerobic metabolism in various organelles such as chloroplast, mitochondria, and plasma membrane (Sharma et al., 2012); chloroplast has been regarded as the main site producing ROS due to photosynthesis. ROS, if not scavenged by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate (APX), can attack diverse cellular organelles, leading to lipid peroxidation or decline in function of various cellular organelles (Noctor and Foyer, 1998; Lukatin 2001; Gülen et al., 2008).

ROS, including superoxide and hydrogen peroxide has been quantified conventionally by histochemical staining techniques. Superoxide anion is stained with nitroblue tetrazolium (NBT) and hydrogen peroxide by Diaminobenzidine tetrahydrochloride (DAB) (Jambunathan, 2010). Histochemical staining for superoxide anion in excised leaves is based on the capacity of cells to reduce NBT (Jabs et al., 1996); NBT particularly react with superoxide and hence, generating a purple or blue formazan precipitation (Doke, 1983). Hydrogen peroxide generally reacts with DAB, thus forming brown polymerization product.

Additionally, thiobarbituric acid (TBARS) assay is routinely employed to determine lipid peroxidation. During the process of lipid peroxidation, the malondialdehyde (MDA) is produced by the decomposition of polyunsaturated fatty acid that can react with thiobarbituric acid (Dhindsa et al., 1981; Yagi, 1998; Jambunathan, 2010).

### ***Critical factors impinging on plant freezing tolerance***

Under environmental and experimental conditions, various critical factors existed directly influencing on plant freezing tolerance, not to mention *freezing per se*. One of such critical factors is thawing (Weiss and Palta, 1991) as cell/organelles relatively rapidly rehydrate following freeze desiccation and cytorrhysis. For instance, the rate of rehydration (thawing) could be a crucial factor in degree of freeze-thaw injury (Yoshida and Sakai, 1968),

while cellular membrane contraction and expansion during freeze-thaw cycles, selectively, could induce freeze-thaw lesions as interactively (Wiest and Steponkus, 1978).

Also, depending upon the cooling rates, the extents of freeze-thaw injury would be different; for example, quick cooling rates (approximately  $3\text{ }^{\circ}\text{C/h}^{-1}$ ) resulted in drastically more severe freeze-thaw lesions than the relatively slow cooling rates (approximately  $1\text{ }^{\circ}\text{C/h}^{-1}$ ) in freeze-thaw injured potato leaflets notwithstanding the freezing temperature eventually attained under two conditions (Steffen et al., 1989). Similarly, the ‘duration of freezing’ (short versus long) at the same temperature may be a one of the critical factors influencing on degree of freeze-thaw injury within plant cells, however, this aspect of freezing tolerance has not been well investigated.

### ***Importance of artificial freeze-thaw protocols and duration of freezing***

Plant cold hardiness is defined as the ability to resist low temperature stress (Levitt, 1980) and is routinely determined as the  $LT_{50}$ , a lethal temperature causing 50 % plant killed, using artificial freeze-thaw protocols; in these protocols plant tissues are generally cooled down at the rate of  $1 \sim 2\text{ }^{\circ}\text{C/h}^{-1}$  to a series of designed treatment temperatures, thawed overnight ( $\sim 12\text{h}$ ), and analyzed, most widely, through the ion-leakage-based analysis, thus resulting in injury (%); percent injury values are calculated from percent ion leakage data (Lim et al., 1998). Most typically artificial freeze-thaw protocols only measured freezing tolerance of excised tissues (green leaves) without considering the whole plant or environmental factors following a freezing episode such as moisture, soil temperature, and wind speed in experimental conditions (Gusta and Wisniewski, 2013); it is, however, difficult to manage all environmental factors, but controllable factors, at least, should be considered to simulate a natural frost episode. One of such controllable factors in a laboratory condition is “duration of freezing” that can play a critical role in evaluating freeze-thaw injured tissues,

(Gusta and Wisniewski, 2013) since the ability of a plant to survive a short-term versus long-term freezing is significantly different (Pomeroy et al., 1975). Moreover, temperatures interpreted as “sub-lethal” based on  $LT_{50}$  actually become lethal depending upon how long it stays cold; for example, the  $LT_{50}$  for DE triticale seedling was  $-23\text{ }^{\circ}\text{C}$ , but was killed at  $-15\text{ }^{\circ}\text{C}$  when frozen for 15 days (Gusta et al., 1997). This notion later was reinforced by Waalen et al. (2011) who suggested that while a conventional freeze-thaw protocol (i.e. cooling rate of  $1\sim2\text{ }^{\circ}\text{C}/\text{h}^{-1}$  at each test treatment temperature) may not be useful to detect small differences in freezing tolerance of winter annuals, exposure them to prolonged freezing at given temperatures is able to reveal which winter annual cultivar is more tolerant. Regardless of its importance, a review and systematic study on “effect of duration of freezing” was unavailable for a relatively less freeze-hardy annual species before the beginning of this thesis.

Taken together, artificial freeze-thaw protocols should be developed and designed in order to reflect and simulate a natural frost episode that may result in new knowledge unknown toward the mechanism of plant freezing tolerance.

### ***Capacity of post-thaw recovery under duration of freezing***

While the cellular and/or molecular mechanisms toward plant cold hardiness has been well investigated, the capacity of plant recovery following freeze-thaw cycle has been largely ignored among botanists. Arora and Palta (1988, 1991) first noted that leaked ions were transported back into the intracellular from extracellular space following post-thaw periods in moderately freeze-thaw injured onion cells that is coincident with a recovery of the plasma membrane as increase in  $\text{H}^{+}$ -ATPase activity. In recent years, Chen and Arora (2013) showed that spinach frozen at a relatively warmer temperature than the  $LT_{50}$  recovered following post-thaw periods through decreasing in leaked ions, reducing in accumulated ROS,

and restoring PSII activity; additionally, molecular chaperones (*S. oleracea* HSP70s and an 85 kDa spinach dehydrin) may be recruited since their expression was observed during post-thaw recovery periods.

The ability of plants to survive a frost episode does depend on not only the resistance / tolerance to perturbations during freeze-thaw cycles, but also their ability to recover from lethal lesions during post-thaw periods (Chen and Arora, 2013). Therefore, the capability of plant post-thaw recovery via short-term versus prolonged freezing at a sub-lethal temperature would be necessarily investigated since temperatures regarded as “sub-lethal” based on LT<sub>50</sub> could be either recoverable or irrecoverable under duration of freezing.

### ***Spinach (Spinacia oleracea L.)***

The plant selected for this thesis is spinach (*Spinacia oleracea* L. cv. Bloomsdale), belongs to a cool-season horticultural crop that has high nutritional and commercial value. The United States is the second largest producer of spinach in the world, but intermittent spring frost can induce substantial loss of spinach yields, which are required to investigate effect of duration of freezing at sub-lethal temperatures on spinach’s freezing tolerance and its capacity of recovery.

As compared with other annual horticultural crops, spinach has relatively lower LT<sub>50</sub>, between -5 and -6 °C (Guy et al., 1987) which provides sufficient sub-freezing ‘temperature treatments’ for the present study.

## Objectives and Significances

The main objective of this thesis is to explore the effect of duration of freezing in spinach leaves frozen at two sub-lethal temperatures, relatively warmer temperatures than its LT<sub>50</sub>. We slightly modified a laboratory-based freeze-thaw protocol for prolonged freezing test; accordingly, samples exposed to various duration of freezing at sub-lethal temperatures were analyzed through diverse physiological parameters: 1) electrolyte ion-leakage, 2) Fv/Fm ratio (efficiency of photosystem II), 3) malondialdehyde (MDA), an indicator of lipid peroxidation, and 4) histochemical detection of reactive oxygen species (ROS), especially of superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). We also investigated the physiological capacity of post-thaw recovery through electrolyte ion-leakage analysis in spinach leaves frozen at a given sub-lethal temperature with various duration of freezing.

The information gained from this thesis will be valuable to further studies on molecular and genetic mechanism of duration of freezing in spinach. Moreover, results from this study may provide new perspective into designing appropriate freeze-thaw protocols in laboratory. On the practical level, such information may be useful to farmers in terms of what to expect or when to protect their spinach under a given frost forecast.

## Thesis Organization

This thesis consists of one journal-styled manuscript preceded by a general introduction (Chap. 1) and followed by a general conclusion (Chap. 3) and appendix. The manuscript was published in *Environmental and Experimental Botany*. The content (i.e. text and figures) of Chapter 2 is identical to the final submission of the manuscript except for necessary formatting changes in order to meet the standard of the graduate college for thesis. Kyungwon Min was the primary researcher for this work under the supervision of Dr. Rajeev

Arora and is the first author of the manuscript.

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## **CHAPTER 2: EFFECT OF SHORT-TERM VERSUS PROLONGED FREEZING ON FREEZE-THAW INJURY AND POST-THAW RECOVERY IN SPINACH: IMPORTANCE IN LABORATORY FREEZE-THAW PROTOCOLS**

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## Abstract

‘Duration of freezing’ at a given treatment temperature can significantly influence the laboratory evaluation of plant freeze-thaw stress tolerance (FTST) by potentially affecting the extent of freeze-thaw injury and, therefore, also the ability of post-thaw recovery. The objective of this study was to compare the extent of freeze-thaw injury, estimated by various physiological indicators [electrolyte leakage, photosystem II efficiency ( $F_v/F_m$ ), accumulation of reactive oxygen species ( $O_2^{\bullet-}$  and  $H_2O_2$ ), and malondialdehyde (MDA) accumulation], in the leaves of spinach (*Spinacia oleracea* L.) when exposed for various durations (0.5, 3.0, 5.5, and 10.5 h) of freezing. Leaf  $LT_{50}$  was first determined (approximately  $-5.5$  °C) and two sub-lethal temperatures,  $-4.0$  and  $-4.5$  °C were selected as the freezing stress. The ability of post-thaw recovery was compared in leaves stressed for shorter versus longer durations at  $-4.5$  °C. All measurements indicated that injury progressively increased with longer freezing durations. While no injury was observed (based on the electrolyte leakage) at  $-4$  °C for 0.5 h, tissues were ~50% injured by the same temperature when frozen for 10.5 h. Moreover,  $-4.5$  °C stress appears as sub-lethal at 3 h of freezing (~30% injury), but, injury doubled (and exceeded the  $LT_{50}$  threshold) after additional 2.5 h at this temperature. Finally, leaves were identically injured to a recoverable point (~ 30 % injury) when frozen for 3 h at either  $-4$  °C or  $-4.5$  °C while 5.5 h of freezing at  $-4.5$  °C caused irreversible injury but no change for  $-4.0$  °C. Our results and corresponding discussion highlight the importance of considering ‘duration of freezing’ as one of the factors for objective interpretation of FTST /  $LT_{50}$  data derived from artificial freeze-thaw tests.

**Keywords:** Duration of freezing, *Spinacia oleracea*, Freeze-tolerance, Oxidative stress,  $LT_{50}$ , Post-thaw recovery

## Introduction

Temperature-controlled, laboratory-based freeze-thaw assays are routinely used to determine cold tolerance of excised tissues in diverse plant species. Typically in these tests, tissues are cooled to a series of sub-freezing temperatures following ice-nucleation, held at respective temperatures for a prescribed duration, thawed at 0 - 4 °C for approximately 12 - 20 h, and subsequently evaluated for freeze-thaw injury using an electrolyte-leakage-based assay; methods other than ion leakage to quantify injury have also been used though much less frequently. The freeze-thaw stress tolerance (FTST) is then determined as LT<sub>50</sub>, the temperature resulting in 50% injury or plant kill (Lim et al., 1998, and references therein); sub-freezing temperatures warmer than LT<sub>50</sub> are, therefore, conventionally regarded as a ‘sub-lethal’ stress. While the importance of using relatively slow cooling and thawing in artificial freeze-thaw assays in order to simulate an episodic natural frost (Steffen et al., 1989; Weiss and Palta, 1991) has been generally recognized, these tests often ignore other critical factors associated with freezing in nature (Gusta and Wisniewski, 2013); these are (among others), administering ice nucleation at relatively warm sub-zero temperatures (Steffen et al., 1989), presence of light (and its intensity) during thaw (Steffen and Palta, 1989; Nezami et al., 2012), frost-injury in dry versus wet tissues (Wisniewski et al., 2002; Gusta et al., 2004; Aryal and Neuner, 2010), potential for a post-thaw recovery (Palta et al., 1977; Arora and Palta, 1991; Chen et al., 2013; Chen and Arora 2013).

Additionally, laboratory freezing tests typically do not consider a potential effect of the ‘duration of freezing’ on the estimated LT<sub>50</sub> since tissues are conventionally exposed to test temperatures for a fixed and rather short duration (~30 min to an hour). That prolonged versus short duration of freezing can result in significantly different injury was first reported by Pomeroy et al. (1975) in winter wheat. Roberts (1985) later suggested that prolonged exposure to sub-lethal temperatures was the major cause for winter-kill in winter wheat.

Therefore, LT<sub>50</sub> of a given tissue may greatly vary as a function of the ‘duration of freezing’ employed in artificial freezing protocols. By extension this also suggests that a freezing temperature interpreted to be sub-lethal based on the LT<sub>50</sub> could actually be lethal depending upon the duration of freezing. Gusta et al. (1997) supported this notion for winter-hardy species using several cultivars of winter annuals (LT<sub>50</sub> ranging from -18 to -30 °C). However, no such systematic study is available for a relatively less freeze-hardy, herbaceous annual.

Spinach (*Spinacia oleracea* L.), an important horticultural crop, can experience intermittent spring frost causing injury and economic loss of production. Therefore, it may be useful to study the cellular physiology of FTST as a function of duration of freezing in spinach. And since depending upon the degree of initial freeze-thaw stress a tissue could either be reversibly or irreversibly injured (Palta et al., 1977; Arora and Palta, 1991; Chen et al., 2013; Chen and Arora 2013), it would also be useful to examine the ability of post-thaw recovery by spinach vis-à-vis short versus prolonged freezing at a sub-lethal temperature. .

Several independent investigations of the cellular mechanism of freeze-thaw injury indicate the plasma membrane to be an early locus of injury (Palta and Li, 1980; Steponkus, 1984; Yoshida, 1984). Kendall and McKersie (1989) provided evidence for freeze-thaw to cause oxidative stress, i.e. accumulation of reactive oxygen species (ROS), such as superoxide (O<sub>2</sub><sup>•-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). If not scavenged, ROS can cause damage to membrane lipids (peroxidation of fatty acids) and proteins (denaturation) (Fridovich, 1991, Mittler, 2002), leading to perturbations in membrane structure and function. Also, the declined photosynthesis and compromised photosystem II efficiency have been reported in freeze-thaw injured plant tissues (Rumich-Bayer and Krause, 1986; Steffen et al., 1989; Binder and Fielder, 1996; Rizza et al., 2001; Equiza and Franko, 2010).

In the presented study, our objectives are two-fold: **1)** to explore the hypothesis that increasing freezing duration at a given sub-lethal temperature causes significantly greater



injury to spinach leaf tissues as quantified by various physiological parameters, i.e., electrolyte-leakage, extent of water-soaking, PSII efficiency ( $F_v/F_m$ ), malondialdehyde (MDA) accumulation (an indicator of lipid peroxidation) (Taulavuori et al., 2001; Campos et al., 2003), and accumulation of ROS ( $O_2^{\bullet-}$  and  $H_2O_2$ ); and **2**) to test a hypothesis that tissues frozen for a longer duration at a given temperature are less able to recover during post-thaw compared to those stressed for relatively shorter duration at the same temperature. An electrolyte-leakage-based, temperature-controlled freeze-thaw test was used to first determine the  $LT_{50}$  of spinach leaves and, accordingly, two ‘sub-lethal’ stress temperatures were selected for this investigation.

## Materials and Methods

### *Plant Material*

Spinach (*S. oleracea* L. cv. Bloomsdale) seeds (Stokes Seeds, Inc., Buffalo, NY) were sown in Sunshine LC-1 mix (Seba Beach, Alberta, Canada) and placed in a growth chamber at 15 °C under  $\sim 250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  average photosynthetically active radiation (PAR) and 12 h photoperiod. Seedlings were watered as needed. Two weeks from sowing, temperature was elevated to 20 °C and from this point onwards 150 ppm EXCEL (Scotts Sierra Horticultural Products Company, Marysville, OH) nutrition solution was applied at weekly intervals. Four-week old seedlings (i.e. typically after the second EXCEL application) were used for various experiments described below.

### *FTST ( $LT_{50}$ ) test*

FTST of ‘Bloomsdale’ spinach leaves was determined using the ion-leakage-based temperature-controlled freeze-thaw protocol as detailed in Chen and Arora (2013). Essentially,

a pair of leaves were placed in a 2.5 x 20 cm test tube containing 150  $\mu$ l deionized water and slowly cooled (1  $^{\circ}$ C/ h) in a glycol bath (Isotemp 3028; Fisher Scientific, Pittsburgh, PA) to various treatment temperatures following ice-nucleation at -1  $^{\circ}$ C. Tissues were held for ~30 min at each temperature and thawed on ice overnight. Unfrozen control (UFC) leaves were maintained at 0  $^{\circ}$ C throughout the freeze-thaw cycle. Freeze-thaw tests were independently repeated thrice with each including five replications per treatment temperature; each replication constituted two leaves per /test tube. Percent injury values were calculated from percent ion leakage data (Lim et al., 1998); percent injury data from three freeze-thaw tests were pooled to obtain means and standard errors. LT<sub>50</sub>, the temperature at which tissues reach their 50% of maximum injury, also defined as FTST, was calculated from injury versus temperature sigmoid curve according to Lim et al. (1998).

#### *Short- and long-duration freezing*

Tissues were frozen (as above) at two selected temperatures, -4 and -4.5  $^{\circ}$ C, for four durations, namely 0.5, 3.0, 5.5, and 10.5 h; these samples are marked as injured (INJ) throughout this manuscript. Samples were removed from the freezing bath after desired freezing durations and thawed as described above. Percent injuries corresponding to various freezing durations across two treatment temperatures were calculated as above from the pooled data of four independent freeze-thaw tests, each including five replications per temperature. Additional 8-10 replications per treatment temperature per duration were included in successive experiments to be used for the following measurements:  $F_v/F_m$ , MDA content, and/or ROS accumulation. Four to 6 leaves from these additional replications of INJ samples were selected only from the -4.5  $^{\circ}$ C treatment to record the extent of water soaking (WS) across four freezing durations.

$$F_v/F_m$$

The Chlorophyll fluorescence parameters,  $F_v$  (variable fluorescence) and  $F_m$  (maximum fluorescence) were measured with a chlorophyll fluorometer (PAM-2000, Walz, Effetrich, Germany) in the UFC and INJ samples, as described in Chen and Arora (2013). Measurements were independently repeated thrice with two to three technical replications (two leaves per replicate) per duration per treatment temperature.  $F_v/F_m$  data across all runs and replicates were pooled to calculate means and standard errors.

#### *Malondialdehyde (MDA) content*

MDA content in the UFC and INJ samples was measured using protocols of Dhindsa et al., (1981) and Shi et al. (2006), with some modifications. Samples were ground into fine powder with liquid nitrogen, and 100 mg tissue per treatment (duration as well as temperature) was mixed with 1.5 mL cold 10 % trichloroacetic acid. Samples were vigorously vortexed before centrifugation at 10,000 g for 20 min at 4 °C. Supernatants were divided into three technical replicates of 400 µl and each mixed with an equal volume of 0.5 % 2-thiobarbituric acid. Mixtures were then heated at 95 °C for 30 min followed by cooling on ice for 5 min and centrifugation at 10,000 g for 5 min at room temperature (~20 °C). The absorbance of clean supernatant was measured at 450, 532 and 600 nm using a Beckman UB-DU 520 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). The MDA concentration was calculated by using the formula:  $[MDA] = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$ . The concentration values were converted to nmoles per g. fresh weight for plotting the data. These experiments were independently repeated four times with four to five technical replicates per duration per temperature; leaves from two to three of the technical replicates (i.e. 4 to 6 leaves) were pooled to constitute a sample for MDA extraction resulting in actually 2 technical replicates / temperature/duration per freeze-thaw run. Data were pooled across four

experiments to calculate means and standard errors.

*ROS staining (superoxide and hydrogen peroxide)*

Superoxide ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ) distribution were visualized by nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) staining, respectively as reported by Ramel et al., (2009), Bournonville and Díaz-Ricci (2011) and Jambunathan (2010), with some modifications. For superoxide ( $O_2^{\bullet-}$ ) staining, tissues were immersed in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 % (w/v) NBT and 10 mM  $NaN_3$ . Samples were subjected thrice to a 2-min vacuum infiltration at 100kPa followed by a 10-min vacuum infiltration. Samples were then bleached with acetic acid: glycerol: ethanol (1:1:3; V/V/V) at 95 °C for 20 min, and cooled at room temperature (~20 °C) for 5 min. All samples were given a quick rinse in bleaching solution before photography.

For hydrogen peroxide ( $H_2O_2$ ) staining, tissues were immersed in DAB solution (1mg / ml, pH 3.8) followed by four 2-min vacuum infiltrations at 100 kPa. Subsequently, samples were shaken in DAB solution at 150 rpm for long enough to allow a total incubation of 4 h in DAB solution. Stained samples were bleached with acetic acid: glycerol: ethanol (1:1:3; V/V/V) at 95 °C for 20 min and cooled at room temperature (~20 °C) for 5 min. Samples were quickly rinsed with bleaching solution before photography.

Staining for  $O_2^{\bullet-}$  and  $H_2O_2$  was independently repeated twice with two replications (two leaves / replicate) per duration per temperature.

*Measurement of post-thaw recovery*

Based on the estimation of FTST ( $LT_{50}$ ), a sub-lethal temperature (i.e. relatively warmer than  $LT_{50}$ ) was selected for post-thaw recovery experiments; this approach has been successfully employed earlier for this spinach cultivar (Chen and Arora, 2013). Leaves were

frozen (to  $-4.5\text{ }^{\circ}\text{C}$ ) and thawed as described earlier under ‘FTST test’. Ion-leakage from the INJ samples was measured right after thaw (RAT), along with corresponding control (0D-UFC). A separate set of replicated INJ samples was allowed to recover at  $5 \sim 7\text{ }^{\circ}\text{C}$  (with 12 h photoperiod;  $\sim 50\text{ }\mu\text{moles m}^2\cdot\text{s}^{-1}\text{ PAR}$ ) for 6-d (6D-REC) in the same tube where they were frozen, as per Chen and Arora (2013). Corresponding UFC leaves were also exposed to the same recovery environment and durations, thus resulting in a 6D-UFC. Ion-leakage for 6D-REC and 6D-UFC samples was measured as described above. This test was independently repeated thrice with four to six replications per duration. Data from these experiments were pooled to calculate means and standard errors.

All data were analyzed by Student’s *t*-test with R (version 2.14.1, The R Foundation for statistical computing, ISBN 3-900051-07-0), for mean separation associated with freezing temperatures, freezing durations, and recovery.

## Results

### *FTST ( $LT_{50}$ ) freeze-thaw injury after short- versus long-duration freezing*

Leaf tissues were injured from 0 % (minimum) to 96.89 % (maximum). Therefore,  $LT_{50}$  (i.e. temperature causing 48.5 % injury, the mid-point between minimum and maximum), by extrapolation, was  $\sim -5.5\text{ }^{\circ}\text{C}$  as determined by the Gompertz function fitted to percent injury versus freezing temperature curve (inset; Fig. 1A).

Two temperatures relatively warmer than the  $LT_{50}$ , i.e.  $-4.0$  and  $-4.5\text{ }^{\circ}\text{C}$ , were selected as the sub-lethal stress treatments for comparing injury under prolonged freezing durations. Our data indicate that tissues were progressively more injured with increasing freezing durations at both temperatures, with  $-4.5\text{ }^{\circ}\text{C}$  causing more injury than  $-4\text{ }^{\circ}\text{C}$  treatment (Fig. 1B). Data also indicate that while  $-4\text{ }^{\circ}\text{C}$  treatment barely caused any injury (compared to control) at 0.5 h, it approached approximately 50% injury level at 10.5 h.

Similarly, tissues were relatively mildly injured (~17%) when frozen at -4.5 °C for 0.5 h. However, the injury exceeded 50% by prolonged freezing to 5.5 h, with further increase to ~75% after 10.5 h (Fig. 1B).

The degree of visual WS after four freezing durations at -4.5 °C is shown in Fig. 1C. In general, WS increased (leaves appeared progressively darker) with increasing freezing duration.

#### $F_v/F_m$

$F_v/F_m$  value for the UFC leaves was ~0.81 and it progressively decreased with increasing freezing duration for both treatment temperatures (Fig. 2). At 10.5 h,  $F_v/F_m$  values for both temperature treatments were significantly lower than those at 0.5 h sampling. Tissues frozen at -4.5 °C always had somewhat lower  $F_v/F_m$  than those exposed to -4.0 °C (Fig. 2).

#### *MDA content*

MDA content in the unfrozen control (UFC) leaves was ~ 0.9 nmol g FW<sup>-1</sup>. It progressively accumulated with the increasing duration of freezing at both -4.0 and -4.5 °C (Fig. 3). Samples frozen at -4.5 °C always accumulated more MDA than the -4.0 °C stress.

#### *Histochemical detection for ROS ( $O_2^{\bullet-}$ and $H_2O_2$ )*

The visual distribution of superoxide ( $O_2^{\bullet-}$ ) (blue stain) and hydrogen peroxide ( $H_2O_2$ ) (brownish stain) in UFC and INJ samples is shown in Fig. 4.  $O_2^{\bullet-}$  and  $H_2O_2$  progressively accumulated with increasing duration of freezing at both temperatures. Although the staining intensity was not digitally quantified, ROS accumulation appears higher at -4.5 °C than at -4 °C (compare Fig. 4 A and C with Fig. 4 B and D).

### *Post-thaw recovery*

The ability of post-thaw recovery from freeze-thaw injuries at  $-4.5^{\circ}\text{C}$  under four freezing durations were compared by ion-leakage assessment (Fig. 5);  $-4^{\circ}\text{C}$  treatments were not studied for the recovery. Injury progressively increased from 14.4 % to 30.1 % to 50.4 %, and finally to ~67 % at 0.5, 3.0, 5.5, and 10.5 h, respectively. Tissues frozen for 0.5 h and 3 h showed 60–70% reduced injury after a 6-D recovery period compared to right after thaw (RAT) samples (i.e.,  $14.4\% \pm 3.3$  to  $5.4\% \pm 1.2$  and from  $30.1\% \pm 6.0$  to  $12.4\% \pm 4.0$ , respectively). In contrast, tissues frozen for 5.5 h and 10.5 h exhibited a further 12–20% increase in injury ( $50.4\% \pm 5.7$  to  $56.3\% \pm 6.4$  and  $67.1\% \pm 6.4$  to  $80.5\% \pm 2.1$ , respectively) after an identical post-thaw recovery treatment (Fig. 5). These observations indicate that the relatively shorter freezing (0.5 and 3.0 h) resulted in injury that was reversible while the longer freezing (5.5 and 10.5 h) caused irreversible injury.

## **Discussion**

While significant advances have been made in last few decades towards understanding the biology of plant FTST, one question continues to get debated among researchers: how to evaluate plant FTST under laboratory conditions to properly predict field survival after a natural freeze? In other words, how to simulate a natural frost episode in artificial freeze-thaw protocols? It is generally recognized that it may be unrealistic to expect an accurate simulation of natural frost in the laboratory since it would necessitate, among other things, incorporating multiple, rather difficult-to-manage, environmental factors such as moisture, wind, rhizosphere temperature, ice encasement, radiational cooling. However, it should also be recognized that some controllable factors impinging greatly upon FTST are often ignored in laboratory freeze tests (Gusta et al., 2009). One such factor is the duration of freezing at a given temperature. A few reports have recently highlighted the importance of

this aspect using whole plant freezing assays and concluded that freezing tolerance of certain winter annuals is best revealed by a prolonged freeze test rather than a short-term freezing (Gusta et al., 1997; 2001; Skinner and Garland-Campbell, 2008; Waalen et al., 2011). In contrast, artificial freezing tests that determine  $LT_{50}$  typically expose tissues to treatment temperatures for ~30 - 60 min or less. Results from the present study, employing several physiological indicators of injury in spinach leaves (membrane-transport perturbation, PSII efficiency, ROS production and lipid peroxidation), reinforce the notion that a combined effect of both the degree and the duration of freezing on FTST (or  $LT_{50}$ ) should be considered when interpreting results from artificial freeze tests and / or relating laboratory observations to field survival.

Freeze-thaw test employed in the present study resulted in a  $LT_{50}$  of ~ -5.5 °C, a value in accordance with other reports for this cultivar (Guy et al., 1987; Chen and Arora 2013). Two levels of freezing stress, ~ 1 °C to 1.5 °C warmer than the estimated  $LT_{50}$ , were selected for this study, since these would conventionally be regarded as sub-lethal. When stressed at -4 °C for 0.5 h, leaves were essentially uninjured (no significant difference from UFC) while tissues showed ~50% injury when frozen for 10.5 h at the same temperature (Fig. 1B). This indicates that depending upon the duration of freezing, a temperature otherwise interpreted as being ‘non-injurious’ could indeed become  $LT_{50}$ . This anomaly is further magnified when freezing stress is increased only by 0.5 °C. For example, freezing at -4.5 °C appears sub-lethal when tissues are held for 3h (~30% injury), whereas injury almost doubled (~60%) after an additional 2.5 h. Such differences in injury are also evident in the extent of leaf WS (Fig. 1C); water-soaked appearance results from the thawed water, most of which migrates out of the cell to growing ice crystals and has not yet been re-absorbed. It is worth noting that the other three physiological parameters (i.e.  $F_v/F_m$ , MDA accumulation, and ROS accumulation) all showed some injury at ‘-4 °C + 0.5 h’ treatment while electrolyte leakage



did not (compare Fig 1A with Figs. 2-4). This indicates differential response of various cellular compartments to freeze-thaw stress.

Our data also indicate that while stress experienced by freezing for 3 h at -4 °C and -4.5 °C was almost identical (~30 % injury), tissues at -4.5 °C were twice as injured with additional 2.5 h of freezing but meanwhile no change in injury was observed at -4 °C treatment (Fig. 1B). Moreover, samples frozen at -4 °C and -4.5 °C for 10.5 h sustained roughly the same amount of injury as those frozen for only 0.5 h at -5.5 °C and -6 °C, respectively, (Fig. 1A and B). These results further highlight the importance of the combined effect of the degree and duration of freezing stress on the final injury. In a study with ‘Jersey Giant’ cultivar of *Asparagus officinalis* L., Arora et al. (1992) noted that a substantially higher spear-kill (78 %) in the unprotected field plots (compared to plastic-covered plots; 17 % spear-kill) was attributed to a combined effect of the degree and duration of freezing, since the former were exposed to temperatures 1-1.5 °C colder than the LT<sub>50</sub> and for additional 4-5 h.

Aside from the practical value of these observations in context with a spring or fall frost, discrepancies among laboratories for reported LT<sub>50</sub> or % injury for the same species/tissue could be also attributable, in part, to the differences in ‘freezing durations’ used in freezing tests. Our data also suggest that the LT<sub>50</sub> based on a short-term freezing assay may over-estimate FTST of plant tissues when compared with the LT<sub>50</sub> or plant survival after a longer-term freezing at an identical temperature regime. In support of this notion, Gusta et al. (1997) using whole plants / root trainers in artificial freeze tests (O’Connor et al., 1993) noted that though LT<sub>50</sub> for DE 15 triticale seedlings was -23 °C, exposure to -15 °C for 9.5 d killed the seedlings; in their estimation for LT<sub>50</sub>, plants were removed for thawing immediately after reaching a treatment temperature. Similarly, a prolonged freezing test ranked Norstar winter wheat significantly hardier than Siouxland while both had LT<sub>50</sub> of ~-22 °C. Waalen et al.

(2011) reported that a short-term freeze-test (plants removed to thaw immediately after reaching a test temperature) revealed no differences in the root regeneration ability (post-thaw) of *Brassica rapa* and *Brassica napus*, whereas prolonged freezing test ranked the latter as significantly hardier species using same criterion. Our data highlights that for a relatively less hardy annual species (e.g. spinach), such discrepancies may become profoundly apparent even if freezing durations differ only by 2-3 h, instead of days.

Freeze-thaw stress compromises photosynthetic capacity of green tissues (Hinch et al., 1987; Steffen and Palta, 1987; Steffen et al., 1989; Ehlert and Hinch, 2008), which may be caused by injuries to chloroplast membranes, photosystem reaction centers and / or Calvin cycle enzymes. Reduced PSII efficiency, estimated by chlorophyll fluorescence parameter,  $F_v/F_m$ , has been widely used as an indicator of injury due to abiotic stresses, including freeze-thaw (Binder and Fielder, 1996; Rizza et al., 2001; Percival and Henderson, 2003; Equiza and Francko, 2010). Accordingly, our data indicated progressively declining PSII efficiency of spinach leaves as duration of freezing increased (Fig. 2). Freeze-thaw stress also causes excessive accumulation of ROS, and hence oxidative stress (Kendall and McKersie, 1989; Baek and Skinner, 2012). The assessment of  $O_2^{\cdot-}$  and  $H_2O_2$  distribution and MDA accumulation (a biomarker for lipid peroxidation) indicate that spinach leaves exposed to prolonged freezing sustained greater injury with damage being worse at  $-4.5\text{ }^{\circ}\text{C}$  (Fig. 3 and 4).

Enhanced oxidative injury might be linked to the observed functional deficiency of PSII. Photosynthesis is a well-established source for ROS. Under normal conditions, however, ROS are routinely quenched by cellular antioxidant systems. Compromised PSII efficiency, possibly due to damage to water-splitting complex (Pospíšil, 2009) and limited  $CO_2$  fixation could lead to over-reduction of photosynthetic electron transport system resulting in excessive ROS production (Suzuki and Mittler, 2006). ROS can, in turn, be damaging to PSII via oxidation of D1 protein (Krieger-Liszkay et al., 2008). Oxidative injury are also

manifested at the cellular membranes (by MDA accumulation), possibly resulting in enhanced ion-leakage in INJ samples. Notably, freeze-thaw stress might not only enhance ROS production by disrupting cellular homeostasis but also inhibit the activity of antioxidant enzymes resulting in the rate of ROS production exceeding the capacity of detoxifying systems. We have recently reported a significant reduction in the activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), following a sub-lethal freeze-thaw injury in ‘Bloomsdale’ spinach leaves which also exhibited excessive accumulation of ROS (Chen and Arora, 2013).

The impact of freezing duration is further highlighted here by studying the ability for post-thaw recovery by spinach leaves following short versus prolonged freezing. For example, freezing for 3 versus 5.5 h at -4.5 °C resulted in either a recoverable lesion or irreversible injury (Fig. 5), as assessed by ion-leakage. Data from Fig. 5 and Fig. 1B taken together further suggest that under a prolonged freezing, a stress increased only by 0.5 °C could translate into a sub-lethal (recoverable) versus lethal (irreversible injury) lesion; leaves were identically injured to a recoverable point (~ 30 % injury) after freezing for 3 h at either -4 °C or -4.5 °C, while 5.5 h of freezing at -4 °C caused no change in injury but that at -4.5 °C caused irreversible injury [(approaching 70%, a non-recoverable level earlier reported by Chen and Arora (2013)]. Plasma membrane H<sup>+</sup>-ATPase, a key membrane transport protein, is believed to be an early site of freeze-thaw injury (Hellergrén et al., 1987; Iswari and Palta, 1989; Arora and Palta, 1991; Ryppö et al., 1997), and has been linked to enhanced ion-leakage by injured tissues. Moreover, recovery of the H<sup>+</sup>-ATPase activity coincides with, and may be required for post-thaw recovery (Arora and Palta, 1991; Ryppö et al., 1997). Indeed, Chen and Arora (2013), based on their work with ‘Bloomsdale’ spinach, suggested that recruitment of molecular chaperones (*S. HSP70s* and an 85kDa spinach dehydrin) may be one of the mechanistic components of post-thaw recovery since their expression was restored

during recovery after an initial repression in INJ tissues. Possibly, stresses due to prolonged freezing interfere with such recruitment, causing irreparable injury to  $H^+$ -ATPase.

Cellular dehydration is most widely believed to be the major cause of injury due to extracellular freezing. Osmotically active cellular water migrates to extracellular ice due to vapor pressure gradient till the cell sap concentration attains equilibrium with the freezing temperature, in osmotic pressure terms to  $-1.86\text{ }^{\circ}\text{C}$  per osmolal (Osm) for aqueous solutions (Handbook of Chemistry and Physics). As illustrated in Fig. 6, freezing of 'Bloomsdale' spinach leaves with initial osmotic pressure of  $\sim 0.3$  Osm would result in cell sap concentration of  $\sim 2.4$  Osm when frozen to  $-4.5\text{ }^{\circ}\text{C}$  (efflux of  $\sim 88\%$  of freezable water). Question thus arises: why is injury substantially more at longer duration despite identical dehydration (Fig. 6)? Precise cellular / molecular explanation for this phenomenon remains elusive but possible explanations may be speculated. An evidence-based notion using the protoplast system has been advanced in cold hardiness biology literature that individual lesions to plasma membrane, arguably a primary site of freeze-thaw injury, have specific freezing temperature thresholds (Webb et al., 1994; Uemura et al., 1995). Therefore, it is likely that various possible cellular lesions resulting from the disrupted homeostasis also have limits of 'duration' to tolerate a given dehydration; this proposal, of course, assumes final injury to be additive whereas synergy between lesions is also possible. For example, increased duration of mechanical stress and strain due to extracellular ice and cell collapse causing shearing at the cell membrane-cell wall adhesion sites could amplify the injurious effect of cellular dehydration over time. In-depth investigation is needed to discern the cellular / molecular mechanism of injury due to shorter versus prolonged freezing.

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## Figure Captions

**Fig. 1. (A)** Sigmoid curve for freeze-thaw injury in the leaves of 4-week old spinach (*Spinacia oleracea* L. cv. Bloomsdale) seedlings, generated from percent injury at individual treatment temperatures using Gompertz function;  $LT_{50}$ , a mid-point (48.5%) between the minimum (0%) and maximum (~97%) injury is defined as the temperature causing 50 % injury. **(B)** Percent injury in leaves exposed for various durations to -4 and -4.5 °C. Unfrozen leaves were used as the control (UFC). The values on the graph represent means and standard errors from the pooled data across five independent freeze-thaw assays, each including five replications per treatment temperature; **(C)** Degree of water soaking (WS) in injured leaves exposed to various duration of freezing at -4.5 °C compared to UFC leaves with no water-soaking (NWS).


**Fig. 2.** Changes of maximal quantum yield of photosystem II ( $F_v/F_m$ ) in freeze-thaw stressed leaves of spinach (*Spinacia oleracea* L. cv. Bloomsdale) seedlings as a function of different durations of freezing (0.5, 3.0, 5.5, and 10.5 h) at -4 and -4.5 °C. Unfrozen leaves were used as control (UFC). Values on the graph represent means and standard errors from the pooled data across three independent freeze-thaw assays each with two to three technical replications (two leaves / replicate) per duration per treatment temperature.

**Fig. 3.** Changes of MDA (malondialdehyde) content in freeze-thaw stressed leaves of spinach (*Spinacia oleracea* L. cv. Bloomsdale) seedlings as a function of different durations of freezing (0.5, 3.0, 5.5, and 10.5 h) at -4 and -4.5 °C. Unfrozen leaves were used as the control (UFC). The values represent means and standard errors from the pooled data across four independent experiments, each with two technical replicates; constitution of technical

replicates is explained under Materials and Methods section.

**Fig. 4.** The distribution of superoxide ( $O_2^{\bullet-}$ ) (A and B) and hydrogen peroxide ( $H_2O_2$ ) (C and D) in freeze-thaw stressed leaves of 4-week old spinach (*Spinacia oleracea* L. cv. Bloomsdale) seedlings exposed to various durations of freezing at -4 and -4.5 °C. Superoxide ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ) were visualized by the histochemical staining (as described under Materials and Methods section) as the blue and brown precipitates, respectively. Unfrozen leaves were used as the control (UFC).

**Fig. 5.** Percent injury in freeze-thaw stressed leaves of 4-week old spinach (*Spinacia oleracea* L. cv. Bloomsdale) seedlings (and the corresponding unfrozen controls, UFCs) right after thaw (RAT) and after 6 d of post-thaw recovery (6D-REC); Freeze-stress treatments constituted 4 durations of freezing at -4.5 °C. The data presented are means and standard errors calculated from the pooled data across three independent experiments each with six technical replicates per duration treatment.

**Fig. 6.** A model illustrating substantially different levels of freeze-thaw injury when tissues are stressed for shorter versus longer freezing durations but at a constant freezing temperature / freeze-desiccation. Small versus large size of the symbols, + / - and  represent lower versus higher ion-leakage and water soaking, respectively. Similarly, small versus big font-size of 'cellular dysfunction' represents the relative level of injury under two scenarios. CM, cell membrane; CW, cell wall; Osm, Osmolal

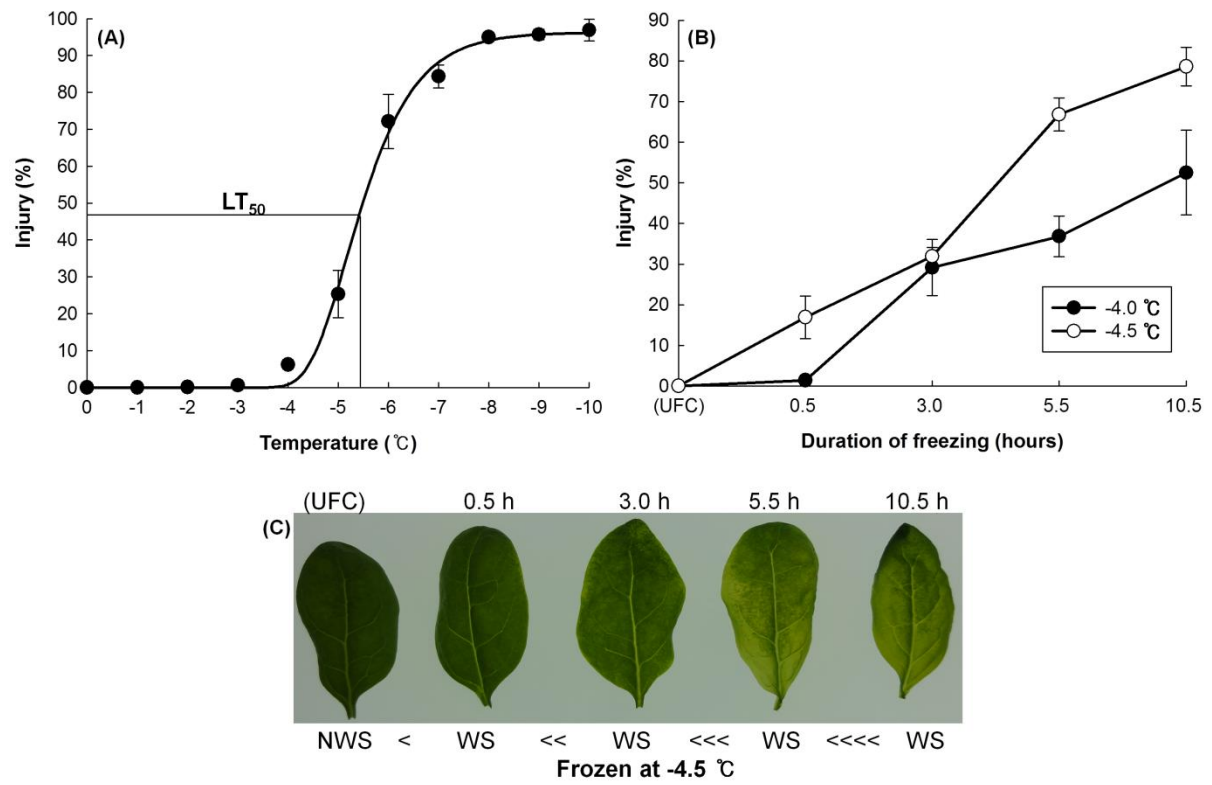


Fig. 1

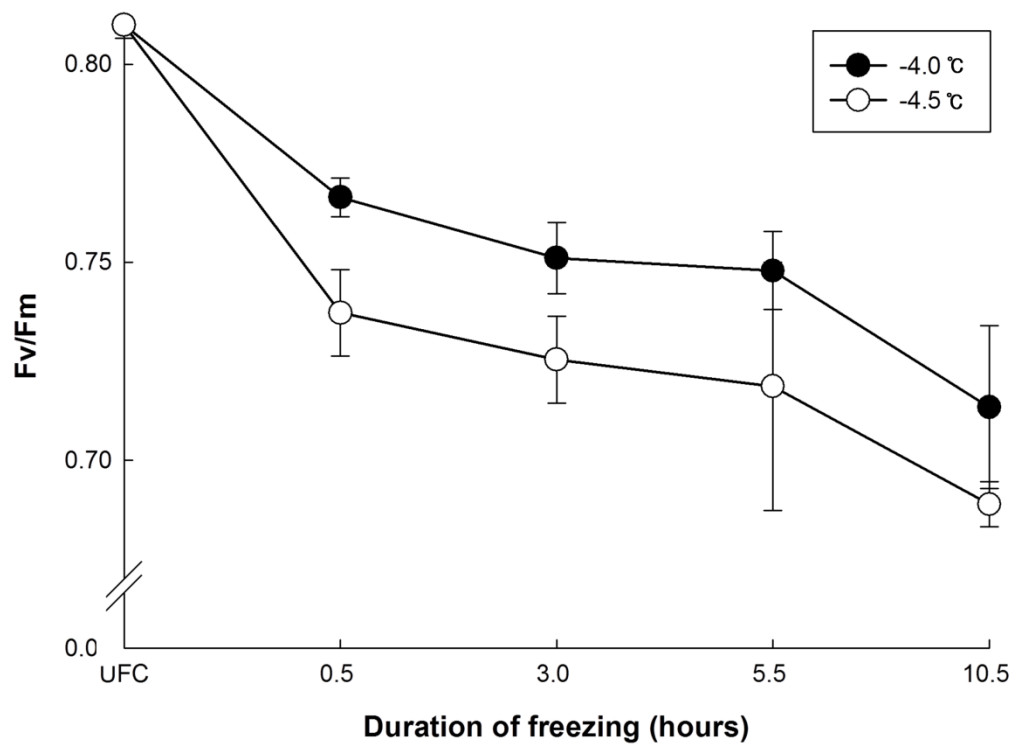


Fig. 2

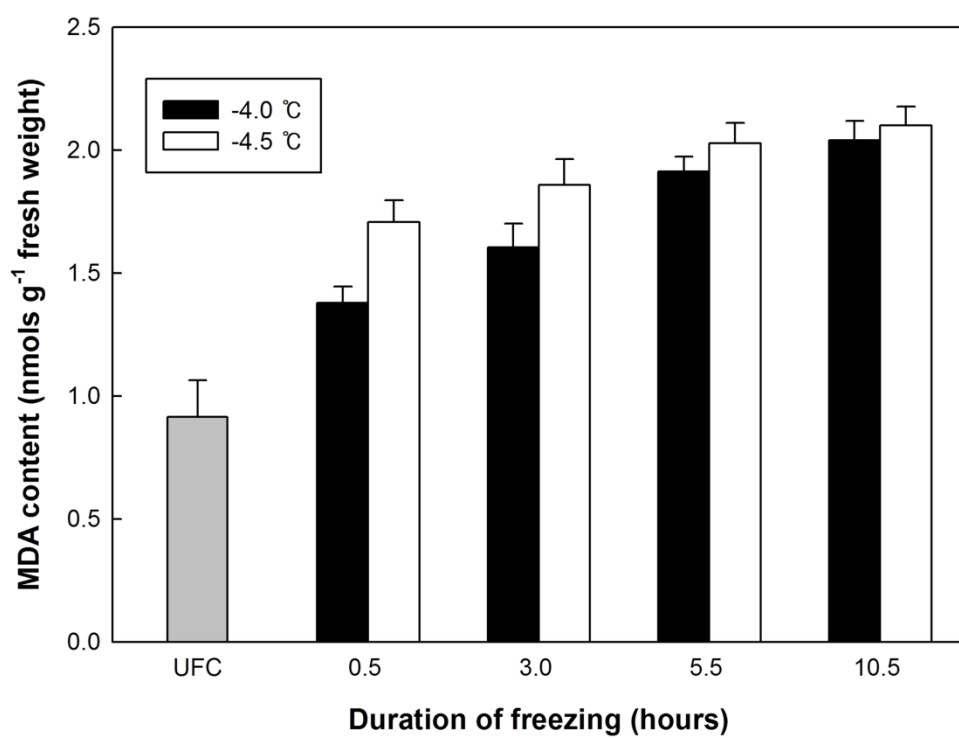


Fig. 3

Fig. 4



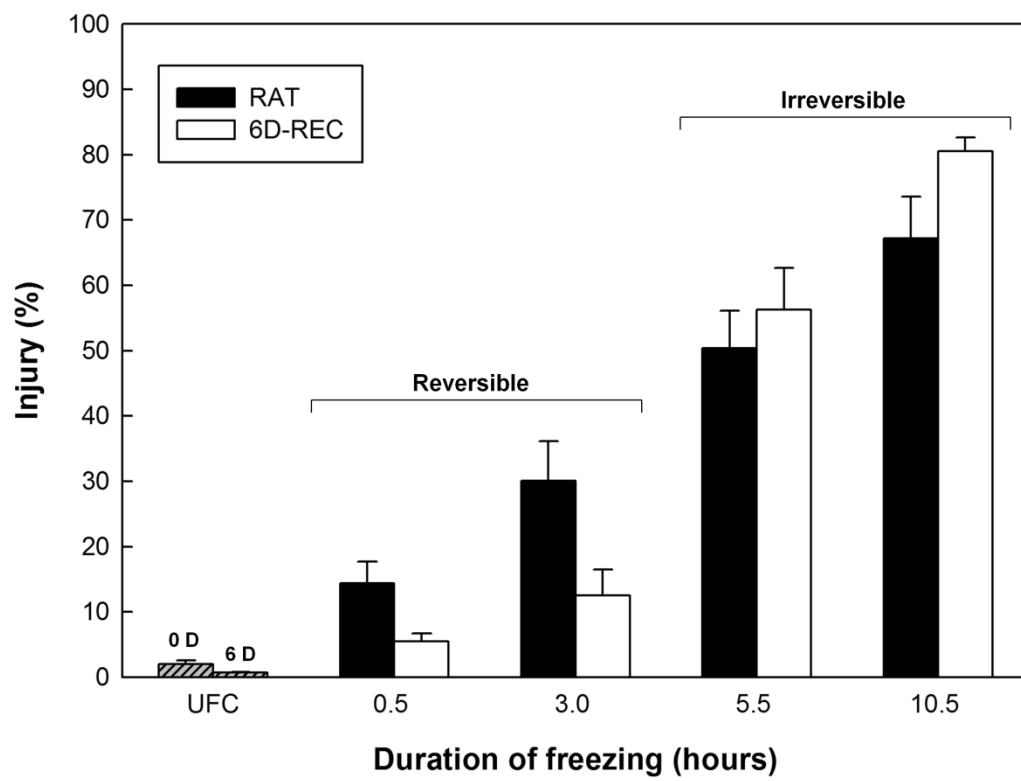


Fig. 5

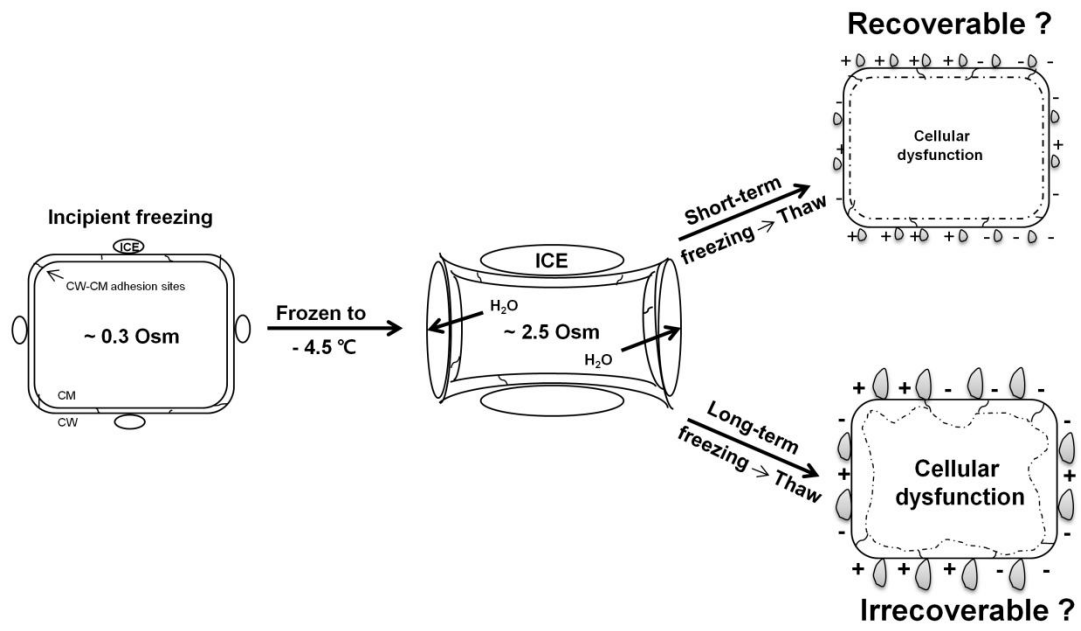


Fig. 6

### CHAPTER 3. GENERAL CONCLUSION

In this project, we studied the physiological basis of freezing tolerance through duration of freezing and ability of post-thaw recovery, using freeze-thaw injured spinach tissues exposed to four durations of freezing at two sub-lethal temperatures. We initially performed the temperature-controlled, electrolyte-based freezing test used widely among researchers to determine the  $LT_{50}$  of spinach (i.e.  $-5 \sim 6$  °C) and therefore,  $-4$  and  $-4.5$  °C were selected as sub-lethal temperatures for spinach (*Spinacia oleracea* L.).

Depending upon duration of freezing, percent injury as based on electrolyte ion-leakage was significantly increased to the threshold of  $LT_{50}$  level when spinach tissues are stressed at  $-4$  °C for 10.5 h while those showed over 50 % when frozen at  $-4.5$  °C for 5.5 and 10.5 h. This indicates that final injury could be determined by the combined influence of degree and duration of freezing. In other words, temperatures interpreted as “sub-lethal” based on  $LT_{50}$  can become actual “lethal” with a longer duration of freezing. That could be reinforced by other data such as decline in efficiency of photosynthesis ( $F_v/F_m$ ), increase in malondialdehyde (MDA) and reactive oxygen species (ROS) in which spinach tissues exposed to a longer duration rather than a shorter duration always showed greater injury with damage being worse at  $-4.5$  °C than  $-4.0$  °C.

Cold temperature has been regarded to inhibit/decrease the enzymatic reactions of photosynthesis such as Calvin cycle enzymes, which potentially is linked to photon flux in excess of that is required for photosynthesis, and hence photo-inhibition, resulting in accumulation of ROS (Öquist and Huner, 2003); these ROS can react with various cellular organelles, including chloroplast and therefore, the function of PSII can be reduced. Reduction in the function of PSII ( $F_v/F_m$  ratio), however, may not be caused by photo-inhibition (over-reduction of photosynthetic transport system) in laboratory conditions, but

caused by inhibited antioxidant enzymes. This is because spinach tissues were not allowed to receive any light energy during freeze-thaw cycles, except for thawing at room temperature (less than  $5 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 40 min); this amount of light energy may not be sufficient to cause photo-inhibition. Therefore, we concluded that altered antioxidant enzymes induced by prolonged freezing may cause increase in ROS during thawing period in experimental conditions.

The impact of duration of freezing can be more reinforced by post-thaw recovery test in freeze-thaw injured spinach leaves. As reported in Chapter 2 (Results from Fig. 5), spinach leaves subjected to relatively short-term freezing (e.g. 30 min or 3.0 h) could be recovered, but not recovered when stressed for 5.5 and 10.5 h at  $-4.5^\circ\text{C}$ . This result indicates that only 2-3h longer duration of freezing at a sub-lethal temperature may cause a relatively less hardy species to be irreversibly injured. Moreover, that recovery from freeze-thaw injury is related to restore  $\text{H}^+$ -ATPase located in plasma membrane was first reported by Arora and Palta (1991). Based on this report, I suggest that a longer duration of freezing may be potentially linked to activity of  $\text{H}^+$ -ATPase being worse or completely dysfunction according to the duration of freezing period. Taken together, as considering all results from our experiment, prolonged freezing to plants at sub-lethal temperature may ultimately cause aggravate activity of all enzymes within plant cells, even makes them no chance to be restored if exposed them to prolonged freezing until threshold at which plants no longer maintain their homeostasis.

In summary, we studied the effect of duration of freezing at sub-lethal temperature via various physiological parameters, using freeze-thaw injured spinach leaves. Through our data, we concluded that duration of freezing, a controllable factor in laboratory condition, should be regarded when evaluating the plant freezing tolerance since plant tissues could get injured 50 % with a longer duration of freezing at sub-lethal temperatures and hence, irrecoverable. In the future, further study of the molecular and genetic mechanism should be

performed in order to understand more in depth with respect to effect of duration of freezing at sub-lethal temperatures.

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## **APPENDIX A**

### **P-values for the Figures in the Thesis**

This appendix provides the p-values for the statistical analyses of the data presented in the Figure 1 (A and B), 2, 3, and 5 of this thesis. In each table, p-values are presented for mean differences among temperature / duration treatment, but recovery duration / freezing duration for Figure 5.

**Supplementary Table 1.** P-values from Student's t-test (calculated by R) for LT<sub>50</sub> measurements corresponding to ten freezing temperatures (-1 through -10) of Figure 1A. UFC, unfrozen control.

	UFC	-1 °C	-2 °C	-3 °C	-4 °C	-5 °C	-6 °C	-7 °C	-8 °C	-9 °C	-10 °C
UFC	---										
-1 °C	0.619	---									
-2 °C	0.247	0.136	---								
-3 °C	0.119	0.108	0.197	---							
-4 °C	0.010	0.011	0.012	0.018	---						
-5 °C	0.013	0.013	0.013	0.014	0.024	---					
-6 °C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	---				
-7 °C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.150	---			
-8 °C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.026	0.028	---		
-9 °C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.052	0.136	0.048	---	
-10 °C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.026	0.026	0.844	0.005	---

**Supplementary Table 2.** P-values from Student's t-test (calculated by R) for prolonged freezing measurements corresponding to two freezing temperatures (-4 and -4.5 °C) and four durations (0.5, 3.0, 5.5, and 10.5 h) of Figure 1B. Treatments are denoted as temperature / duration (e.g. -4 °C / 0.5 h) and / or unfrozen control (UFC).

	-4°C /0.5 h	-4°C /3.0 h	-4°C /5.5 h	-4°C /10.5h	-4.5°C /0.5 h	-4.5°C /3.0h	-4.5°C /5.5 h	-4.5°C /10.5h	UFC
-4°C /0.5 h	---								
-4°C /3.0 h	0.058	---							
-4°C /5.5 h	0.090	0.442	---						
-4°C /10.5h	0.029	0.181	0.327	---					
-4.5°C /0.5 h	0.083	0.234	0.078	0.065	---				
-4.5°C /3.0h	0.007	0.762	0.533	0.213	0.079	---			
-4.5°C /5.5 h	0.039	0.020	0.048	0.360	0.004	0.008	---		
-4.5°C /10.5h	0.003	0.006	0.014	0.136	<0.001	0.001	0.162	---	
UFC	0.009	0.054	0.087	0.027	0.073	0.006	0.038	0.003	---



[illegible]

**Supplementary Table 4.** P-values from Student's t-test (calculated by R) for MDA measurements corresponding to two freezing temperatures (-4 and -4.5 °C) and four durations (0.5, 3.0, 5.5, and 10.5 h) of Figure 3. Treatments are denoted as temperature / duration (e.g. -4 °C / 0.5 h) and / or unfrozen control (UFC).

	-4°C /0.5 h	-4°C /3.0 h	-4°C /5.5 h	-4°C /10.5h	-4.5°C /0.5 h	-4.5°C /3.0h	-4.5°C /5.5 h	-4.5°C /10.5h	UFC
-4°C /0.5 h	---								
-4°C /3.0 h	0.205	---							
-4°C /5.5 h	0.006	0.025	---						
-4°C /10.5h	0.003	0.009	0.307	---					
-4.5°C /0.5 h	0.058	0.456	0.077	0.023	---				
-4.5°C /3.0h	0.011	0.081	0.702	0.247	0.242	---			
-4.5°C /5.5 h	0.002	0.009	0.308	0.979	0.023	0.248	---		
-4.5°C /10.5h	0.001	0.002	0.093	0.535	0.005	0.096	0.564	---	
UFC	0.020	0.003	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	---

**Supplementary Table 5.** P-values from Student's t-test (calculated by R) for post-thaw recovery measurements corresponding to -4.5 °C and four durations (0.5, 3.0, 5.5, and 10.5 h) of Figure 5. Treatments are denoted as recovery duration / freezing duration (e.g. RAT / 0.5 h or REC6D / 0.5 h). RAT, right after thaw; REC6D, recovery for 6 d; UFC-RAT, unfrozen control for injured sample right after thaw; UFC-6D, unfrozen control for 6-d recovered samples.

	RAT /0.5 h	RAT /3.0 h	RAT /5.5 h	RAT /10.5 h	REC6D /0.5 h	REC6D /3.0 h	REC6D /5.5 h	REC6D /10.5 h	UFC- RAT	UFC- 6D
RAT /0.5 h	---									
RAT /3.0 h	0.037	---								
RAT /5.5 h	<0.001	0.022	---							
RAT /10.5 h	<0.001	<0.001	0.066	---						
REC6D /0.5 h	0.026	0.002	<0.001	<0.001	---					
REC6D /3.0 h	0.715	0.025	<0.001	<0.001	0.116	---				
REC6D /5.5 h	<0.001	0.006	0.499	0.246	<0.001	<0.001	---			
REC6D /10.5 h	<0.001	<0.001	<0.001	0.077	<0.001	<0.001	0.002	---		
UFC- RAT	0.004	<0.001	<0.001	<0.001	0.022	0.023	<0.001	<0.001	---	
UFC- 6D	0.002	<0.001	<0.001	<0.001	0.002	0.012	<0.001	<0.001	0.033	---

## APPENDIX B

### Calculation of $LT_{50}$ and Gompertz function for spinach leaves

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This protocol describes how  $LT_{50}$  of spinach is calculated and plotting a sigmoidal curve based on Gompertz function, using Sigma Plot 12 (Systat Software Inc., San Jose, CA, USA). Spinach tissues are subjected to a controlled freeze-thaw regime followed by estimation of injury by measuring electrolyte ion-leakage from freeze-thaw injured spinach tissues as reported by Lim et al. (1988).

#### Leaf freezing tolerance calculated by $LT_{50}$ and Plotting a Sigmoidal Curve

IL: initial ion-leakage FL: final ion leakage

%  $IL_t$  and %  $IL_c$  represent percentage ion leakage at each test treatment temperature and control temperature.

- ◆ Percent ion leakage:  $(\% IL) = IL / FL \times 100$
- ◆ Percent injury  $(\% injury) = (\% IL_t - \% IL_c) / (100 - \% IL_c) \times 100$
- ◆ Percent adjusted injury  $(\% adjusted injury_t) = (\% injury_t / \% injury_{max}) \times 100$ ; here %  $injury_{max}$  is the injury at  $-10^\circ C$ .
- ◆ Adjusted injury: adjust the values from % adjusted injury; for instance, if values are negative, adjust them to 0 % (no injury) while values are greater than 100 %, adjust them to 100 % (maximum injury)
- ◆ The leaf freezing tolerance is represented by  $LT_{50}$ , lethal temperature causing 50 % plant killed
- ◆ Percent injury at each test treatment temperature is plotted as a sigmoidal curve

based on Gompertz function, using Sigma Plot 12 (Systat Software Inc., San Jose, CA, USA)

- ◆ Each test treatment temperature and corresponded percent injury average, including standard errors is written down as X axis and Y axis, respectively on data sheet of Sigma Plot 12 (Systat Software Inc., San Jose, CA, USA)
- ◆ Click the “Create Graph” in section of Graph and select “Scatter Plot” in section of Graph types, followed by XY pair in the section of Data format
- ◆ As described above, select all test treatment temperatures as X axis and all percent injury as Y axis, followed by standard errors
- ◆ Set the scales of both X axis and Y axis (e.g. from 0 to -10 for X axis and from 0 to 100 for Y axis in section of Graph properties which are appeared by clicking the right mouse)
- ◆ Right mouse click on the graph and select the “Fit curve”. Select “Sigmoidal curve” in section of Equation Category and then, “Gompertz, 3 Parameter” in section of Equation Name, followed by “Finish”
- ◆ Represent  $LT_{50}$  using “Draw Box” in section of Tools and then, Paste to PowerPoint Slide if needed to revise

\*In this thesis, adjusted injury % is not used to calculate  $LT_{50}$  (see detail in Chapter 2 of Materials and Methods section for Fig. 1A)

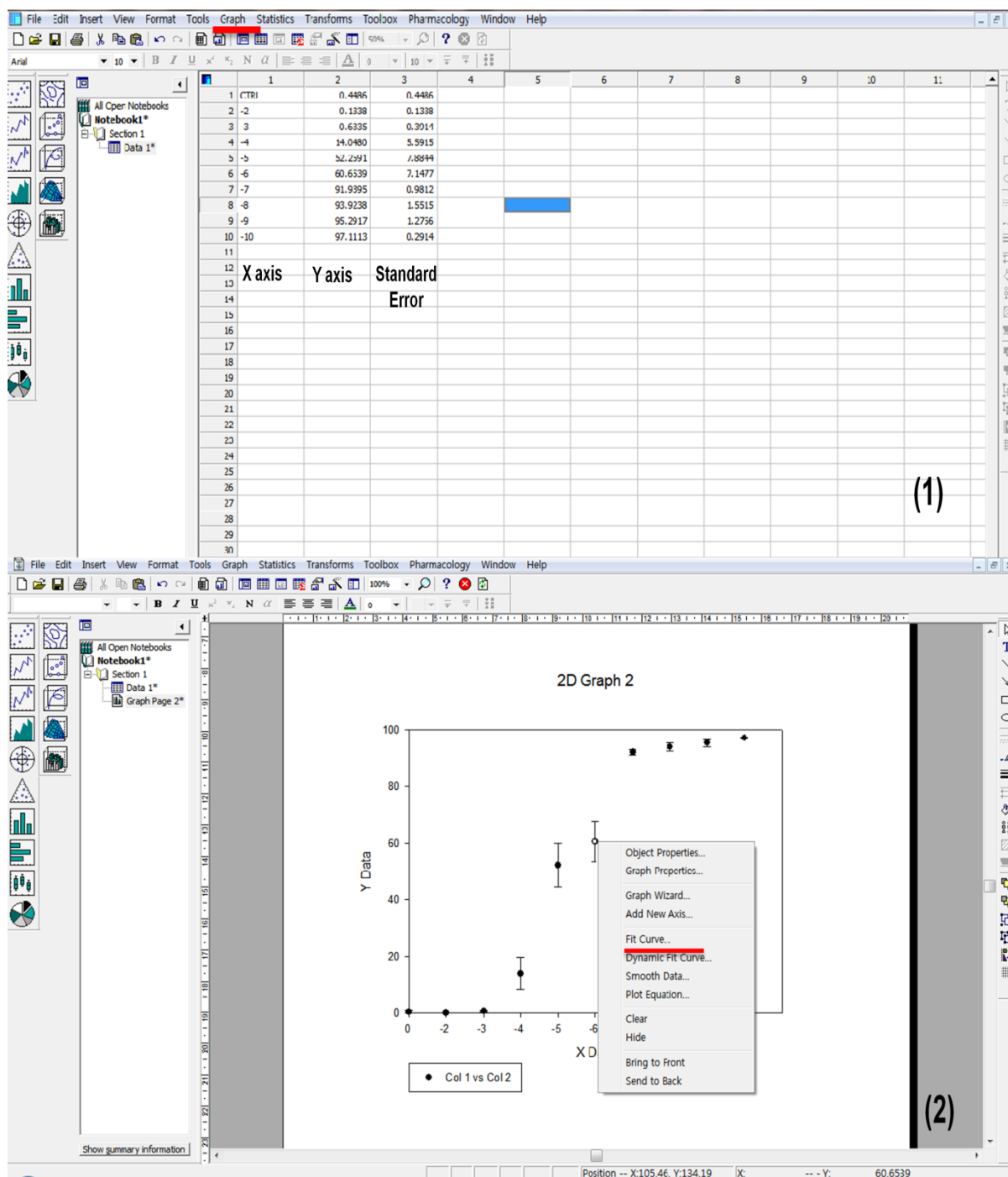
Below is an example data set to illustrate the calculation and plotting a sigmoidal curve for  $LT_{50}$  described above.

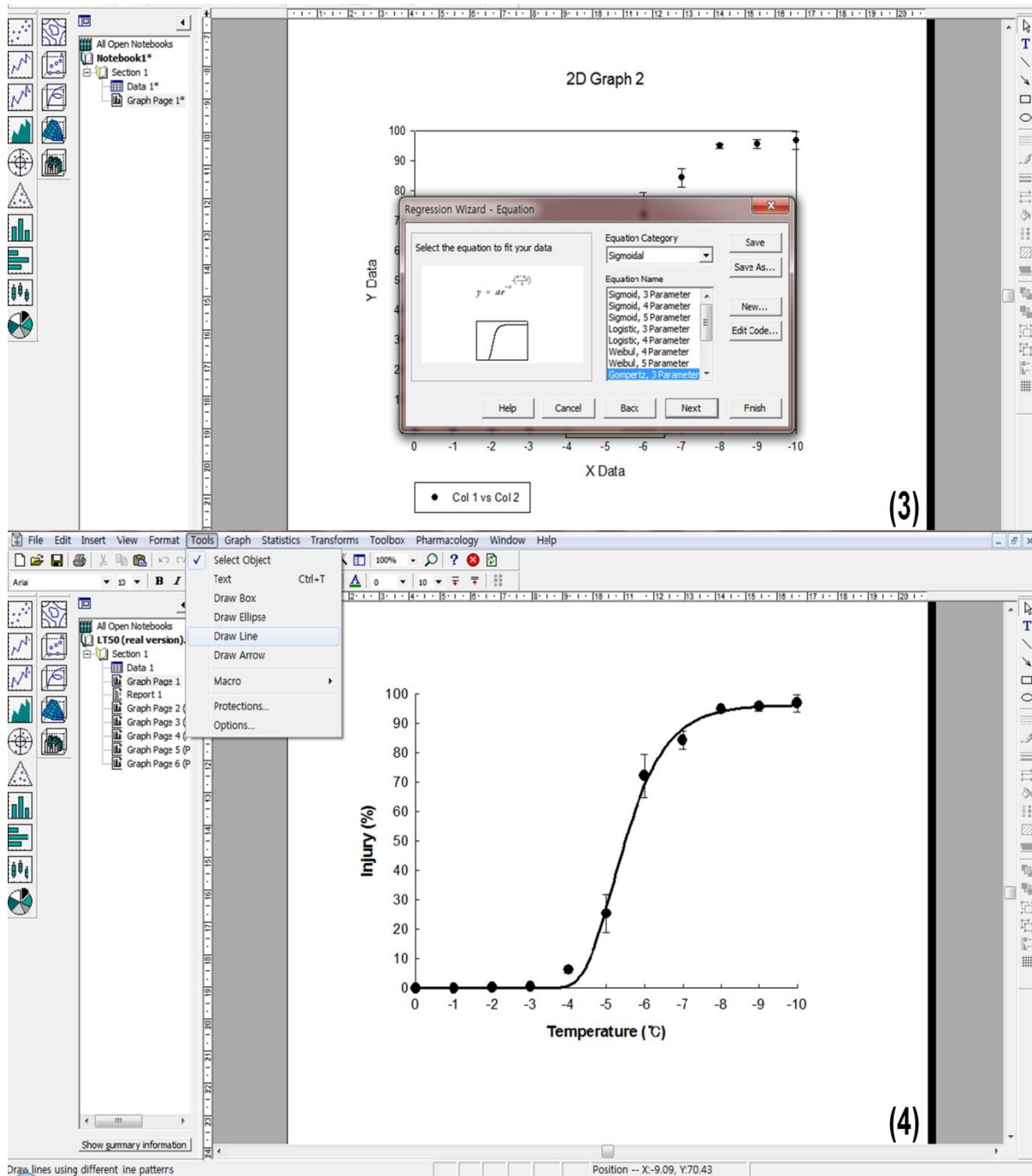
Temp.	IL	FL	% IL	% Injury	% Injury Average	Standard Error	%Adjusted Injury
CTRL A	6.3	406	1.551724	0	0.448623	0.448623	0.461967
CTRL B	14.4	343	4.19825	2.243113			
CTRL C	5	451	1.108647	0			
CTRL D	6.6	383	1.723238	0			
CTRL E	6.5	430	1.511628	0			
-2 A	5.9	470	1.255319	0	0.133842	0.133842	0.137823
-2 B	4.1	408	1.004902	0			
-2 C	5.4	480	1.125	0			
-2 D	5.7	485	1.175258	0			
-2 E	9.8	369	2.655827	0.669211			
-3 A	12	354	3.389831	1.418194	0.633493	0.39145	0.652337
-3 B	14.3	385	3.714286	1.749271			
-3 C	8.3	457	1.816193	0			
-3 D	5	431	1.160093	0			
-3 E	3.6	383	0.939948	0			
-4 A	36.8	438	8.401826	6.532476	14.04796	5.591507	14.46583
-4 B	129	422	30.56872	29.15176			
-4 C	104	387	26.87339	25.38101			
-4 D	47	448	10.49107	8.664359			
-4 E	14	540	2.5	0.510204			

-5 A	298	427	69.78923	69.17268	52.25906	7.884387	53.81356
-5 B	372	503	73.95626	73.42476			
-5 C	205	464	44.18103	43.04187			
-5 D	164	400	41	39.79592			
-5 E	156	420	37.14286	35.86006			
-6 A	160	469	34.11514	32.77055	60.65392	7.14772	62.45814
-6 B	251	392	64.03061	63.29654			
-6 C	308	418	73.68421	73.14715			
-6 D	279	412	67.71845	67.05964			
-6 E	228	337	67.65579	66.9957			
-7 A	326	367	88.82834	88.60034	91.93953	0.981194	94.67438
-7 B	470	504	93.25397	93.11629			
-7 C	461	500	92.2	92.04082			
-7 D	385	407	94.59459	94.48428			
-7 E	383	418	91.62679	91.45591			
-8 A	446	470	94.89362	94.78941	93.92384	1.55148	96.71771
-8 B	415	438	94.74886	94.64169			
-8 C	375	394	95.17766	95.07925			
-8 D	483	497	97.1831	97.12561			
-8 E	442	501	88.22355	87.98322			
-9 A	366	397	92.19144	92.03208	95.29169	1.275607	98.12625
-9 B	476	491	96.94501	96.88266			
-9 C	443	470	94.25532	94.13808			
-9 D	323	343	94.1691	94.0501			

-9 E	472	475	99.36842	99.35553			
-10 A	402	410	98.04878	98.00896	97.11131	0.291411	100
-10 B	363	374	97.05882	96.9988			
-10 C	381	395	96.4557	96.38336			
-10 D	438	449	97.55011	97.50011			
-10 E	444	459	96.73203	96.66533			







## References

Lim C.C., R. Arora, and E.C. Townsend. 1998. Comparing Gompertz and Richards functions to estimate freezing injury in Rhododendron using electrolyte leakage. J. Am. Soc. Hort. Sci. 123:245-252

## APPENDIX C

### Malondialdehyde (MDA) Assay in spinach leaves

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The generation of MDA due to the oxidation of poly-unsaturated fatty acids has been considered as an indicator of lipid peroxidation. This protocol is slightly modified from Chen and Arora (2011).

#### Reagents

◆ **10 % trichloroacetic acid (TCA)**

2 g TCA is dissolved in ddH<sub>2</sub>O (Volume making up to 20 ml). Store at 4 °C

◆ **0.5 % thiobarbituric acid (TBA) containing 20 % trichloroacetic acid (TCA)**

2 g TCA and 0.5 g TBA are dissolved in ddH<sub>2</sub>O (volume making up to 10 ml)

#### Protocol

1. Spinach leaves were ground into fine powder with liquid nitrogen and store at -70 °C
2. Pre-warm the water bath (~ 94 °C) and pre-chill the centrifuge for 15 min at 4 °C.
3. Weigh 100 mg leaf tissue quickly and mixed with 1.5 ml pre-chilled 10 % TCA (use 2.0 ml tubes).
4. Strong vortex and subsequently centrifuge at 10.000g for 20 min at 4 °C.
5. Discard pellet debris, transfer 400 µl supernatant into pre-chilled 1.5 ml tube and subsequently, mixed with 400 µl 0.5 % TBA, thus resulting in three tubes for individual test treatment temperature and/or duration treatment.
6. Vortex the mixture and incubate tubes at ~ 94 °C for 40 min in the water bath (open the caps of all tubes every 10 min otherwise, mixture would be overflowed due to pressure)
7. Turn on the spectrophotometer to warm up for 20 min before next step.

8. Cool down the mixtures on ice for 5 min and centrifuge at 10.000 g for 5 min.
9. Read absorbance at 450, 532, and 600 nm of the mixture in each of the three tubes against a black (everything but no supernatant)

### **Calculation of MDA content**

1.  $[6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}] = A \text{ } [\mu\text{M}]$
2.  $2A \times 1.5 \text{ ml} / 1000 = B \text{ } [\mu\text{Mol}]$
3.  $2A \times 1.5 \text{ ml} \times 1000 / 1000 = C \text{ } [\text{nMol}]$
4. MDA concentration  $[\text{nMol}]$  in 1.5 ml / FW.  $\text{g}^{-1}$  tissue (0.1 g) = D  $[\text{nmol. g}^{-1} \text{ FW tissue}]$

MDA concentration is expressed as nmol MDA per gram of fresh weight. For example, the initial MDA concentration can be calculated according to the formula:  $[6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}] = \text{MDA}$  [step 1]. Since supernatant (400  $\mu\text{l}$ ) was diluted two times by TBA (400  $\mu\text{l}$ ), the MDA concentration gained from [step 1] should be doubled [step 2] and then expressed it as  $\mu\text{Mol}$  for 1.5 ml supernatant. Subsequently,  $\mu\text{Mol}$  is converted as nMol because unit  $[\mu\text{Mol}]$  is too small to express on plotting [step 3] and then divided by 0.1 g (fresh tissue), thus generating final MDA concentration (nmol.  $\text{g}^{-1}$  FW tissue) [step 4].

### **Reference**

Chen, K. and R. Arora 2011. Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in spinach (*Spinacia Oleracea*)